

# Molecular Beacon Reverse Transcription-PCR of Human Chorionic Gonadotropin- $\beta$ -3, -5, and -8 mRNAs Has Prognostic Value in Breast Cancer

PAUL N. SPAN,<sup>1\*</sup> PEGGY MANDERS,<sup>1,2</sup> JOOP J.T.M. HEUVEL,<sup>1</sup> CHRIS M.G. THOMAS,<sup>1</sup>  
REMKO R. BOSCH,<sup>1,3</sup> LOUK V.A.M. BEEX,<sup>2</sup> and C.G.J. (FRED) SWEEP<sup>1</sup>

**Background:** The  $\beta$ -subunit of human chorionic gonadotropin (hCG) is encoded by four genes, of which expression of the hCG $\beta$ -3, -5, and -8 genes could have prognostic value in breast cancer.

**Methods:** Applying a new, modified Molecular Beacon reverse transcription-PCR assay, we investigated the prognostic value of the hCG $\beta$ -3, -5, and -8 gene transcripts in 129 sporadic unilateral breast cancer samples from patients with a median follow-up of 62.3 months.

**Results:** Expression of hCG $\beta$ -3, -5, -8 was significantly ( $P = 0.020$ ) associated with relapse-free survival (RFS). In multivariate survival analysis, hCG $\beta$ -3, -5, and -8 maintained prognostic value for RFS, with high expression predicting shorter RFS ( $P = 0.015$ ; hazard ratio, 2.25; 95% confidence interval, 1.17–4.34). Only 1 of 24 (4%) node-negative patients with low hCG $\beta$ -3, -5, -8 expression relapsed, in contrast to 7 of 26 (27%) patients with high expression ( $P = 0.046$ ).

**Conclusions:** Expression of hCG $\beta$ -3, -5, -8, which differ by only one nucleotide from other hCG $\beta$  genes, can be assessed by our modified Molecular Beacon assay in breast cancer tissues. Expression of hCG $\beta$ -3, -5, -8 has independent, prognostic value for RFS in breast cancer and may help identify node-negative patients with poor prognosis.

© 2003 American Association for Clinical Chemistry

More accurate determination of the prognosis of breast cancer patients contributes to relieving anxiety in those patients who will remain disease free and to adjusting

treatment to improve the predicted outcome in others. The identification of new genes and gene products that can serve as prognosticators in breast cancer may improve the prediction of tumor behavior and may help to reveal molecular events associated with malignant transformation.

Serum human chorionic gonadotropin (hCG)<sup>4</sup> is widely used as a marker for the presence of tumor mass and/or efficacy of therapy in patients with trophoblastic or testicular malignancies (1, 2). Other malignancies, such as bladder (3, 4), pancreatic, and cervical carcinomas (3) and prostate (5, 6) and breast cancer (7–10), also express hCG. This hormone is one of a family of related glycoproteins that includes luteinizing hormone, follicle-stimulating hormone, and thyroid-stimulating hormone. All of these glycoproteins share the same  $\alpha$ -subunit, but their specificity is conferred by the  $\beta$ -subunit. Remarkably, the  $\beta$ -subunit of hCG is encoded by four genes: hCG $\beta$ -3, -5, -7, and -8, located on chromosome 19q13.3 in close proximity to hCG $\beta$ -1 and -2 and the highly homologous gene for luteinizing hormone. The latter was originally designated hCG $\beta$ -4 (11). hCG $\beta$ -1 and -2 are considered pseudogenes. All other genes encode an identical protein, hCG $\beta$ , with the exception that the hCG $\beta$ -7 gene encodes an alanine (GCC) at position 117 compared with an aspartic acid (GAC) encoded by hCG $\beta$  genes -3, -5, and -8. The hCG $\beta$ -7 gene is reportedly the only gene expressed in several nontransformed tissues. The hCG $\beta$ -3, -5, and -8 genes are variably expressed in placental and malignant tissue such as thyroid, prostate, bladder, and breast cancer (8), but not in noncancerous tissues of these organs. The presence or absence of these hCG $\beta$  transcripts

Departments of <sup>1</sup> Chemical Endocrinology, <sup>2</sup> Medical Oncology, and <sup>3</sup> Endocrinology, University Medical Center Nijmegen, 6500 HB Nijmegen, The Netherlands.

\*Address correspondence to this author at: 530 Department of Chemical Endocrinology, University Medical Center Nijmegen, PO Box 9101, 6500 HB Nijmegen, The Netherlands. Fax 31-24-3541484; e-mail p.span@ace.umcn.nl.

Received January 15, 2003; accepted April 25, 2003.

<sup>4</sup> Nonstandard abbreviations: hCG, human chorionic gonadotropin; ER, estrogen receptor; PgR, progesterone receptor; RFS, relapse-free survival; and OS, overall survival.

was found to have independent prognostic value in breast cancer (9).

The highly conserved sequences of these genes make it difficult to differentiate among transcripts expressed by the different hCG $\beta$  genes. Earlier studies used restriction fragment length analysis (12) or nested PCR with primers that differ in one nucleotide at the 3' end (8,9) to differentiate between these transcripts. Recently, we described an assay, modified from the Molecular Beacon principle described by Tyagi and Kramer (13), that was highly specific for the hCG $\beta$ -3, -5, -8 transcripts (6). This modification of Molecular Beacons was later designated "shared stem molecular beacon" by another group (14). With this assay, we aimed to measure the diagnostic accuracy of hCG $\beta$ -3, -5, -8 expression in 129 breast cancer specimens and to assess whether the reported prognostic value of the presence of these transcripts (9) persisted when assessed quantitatively in a separate patient group.

### Patients and Methods

#### PATIENTS

On the basis of the availability of frozen tissue in our tumor bank, we selected a series of 129 women with unilateral, operable breast cancer who underwent resection of their primary tumor between November 1987 and December 1997. This bank contains frozen tumor tissue of patients with breast cancer from five different hospitals of the Comprehensive Cancer Center East in The Netherlands because estrogen (ER) and progesterone receptor (PgR) concentrations measurements for these hospitals were centrally performed in our hospital. The clinical data were retrospectively collected. Patients had no previous diagnosis of carcinoma, no distant metastases at time of diagnosis, and no evidence of disease within 1 month after primary surgery. In addition, patients receiving neoadjuvant therapy or with carcinoma in situ only were excluded. The median age was 57.2 years (range, 30.7–88.3 years). Patients underwent modified radical mastectomy (n = 103) or a breast-saving procedure (n = 26), followed by radiotherapy in 83 patients. A resection was considered complete when there were no tumor cells in the inked border of the histologic section. In case the margin was not free, a re-resection or breast ablation was performed whenever possible or additional radiotherapy was given. Lymph node involvement was found in 74 patients. Subsequent systemic adjuvant therapy (34 endocrine therapy, 10 chemotherapy, 15 both) was given based on the established clinical evaluation criteria of that time. The median follow-up time was 62.3 months (range, 1.2–164.3 months). Patients were seen (history, physical examination, routine laboratory investigations) once every 3 months during the first 2 years, once every 6 months for 5 years, and once a year thereafter. Once a year, x-ray mammography was performed. During follow-up, 43 patients had a recurrence (4 local, 2 regional, and 37 distant metastases), and 32 patients died (27 confirmed

breast cancer-related, 5 unknown). Contralateral breast cancer or second malignancies were not considered as recurrent disease.

#### TISSUE PROCESSING

After primary surgery, a representative part of the tumor was selected by a pathologist, frozen in liquid nitrogen, and sent to our department for routine estrogen receptor (ER) and progesterone receptor (PgR) ligand binding assays as recommended by the European Organization for Research and Treatment of Cancer (EORTC) (15). Tissue aliquots were pulverized by a microdismembrator (Braun) and kept in liquid nitrogen until RNA isolation. Total RNA was isolated from 20 mg of tissue powder with use of the RNeasy Mini reagent set (Qiagen) with on-column DNase-I treatment. The quality of the RNA was checked by examining ribosomal RNA bands after agarose gel electrophoresis and by amplifying  $\beta$ -actin as a control (see below). RNA concentrations were determined from the absorbance at 260 nm (Genequant; Amersham). We found no association between RNA degradation or concentration and length in storage.

#### REVERSE TRANSCRIPTION

Purified total RNA (1.0  $\mu$ g) was denatured for 10 min at 70 °C and immediately cooled on ice. Reverse transcription was performed with the Reverse Transcription System (Promega Benelux B.V.) according to the manufacturer's protocol. After annealing of random hexamers for 10 min at 20 °C, cDNA synthesis was performed for 60 min at 42 °C, followed by an enzyme inactivation step for 5 min at 95 °C.

#### MOLECULAR BEACON DESIGN

A probe (5'-FAM-cgc ttc cag gac tcc aag cg-TAMRA-3', where FAM is 6-carboxyfluorescein and TAMRA is 6-carboxytetramethylrhodamine) that specifically annealed with transcripts from the hCG $\beta$ -3, -5, and -8 genes was designed essentially according to the guidelines for Molecular Beacons described by Tyagi and Kramer (13). However, to obtain a smaller loop sequence, only one arm (shown in italics in the sequence above), complementary to five nucleotides at the 5' end of the hCG $\beta$  sequence, was attached 3' (6,14). This produced a Molecular Beacon-type probe with excellent specificity for hCG $\beta$ -3, -5, and -8 mRNA without interference by transcripts from the hCG $\beta$ -7 or luteinizing hormone genes (6). PCR primers were designed with use of the Primer Express Software (PE Applied Biosystems). Primers were from Biologio, and the probe was from PE Applied Biosystems.

#### GENERATION OF SPECIFIC CALIBRATORS

Specific calibrators for hCG $\beta$ -3, -5, -8 and hCG $\beta$ -7 were generated by primer-mediated mutagenesis. PCR amplification of hCG $\beta$  in the choriocarcinoma cell-line JAR was performed with the following primers: 5'-cac ccc ttg acc

tgt gat gac ccc cgc ttc cag ga/cc tcc t-3' and 5'-gcc taa ctc ttc gga aat aac a-3' (the bold nucleotides represent nucleotides that differ between calibrators). PCR products were cloned into pCR 2.1-TOPO in DH5 $\alpha$  cells with the TOPO-TA cloning reagent set (Invitrogen). Sequences were confirmed by cycle sequencing (AbiPrism 3700; PE Applied Biosystems) with M13 forward and reverse primers. Plasmids were linearized using *Bsp*HI, quantified spectrophotometrically, and diluted to 10<sup>7</sup> copies/ $\mu$ L.

#### PCR

All PCRs were carried out in TaqMan Universal PCR master mixture (PE Applied Biosystems) with 500 nM each primer and 200 nM probe in a final volume of 25  $\mu$ L.  $\beta$ -Actin was amplified with use of Pre-Developed Assay Reagents (PE Applied Biosystems). hCG $\beta$ -3, -5, -8 and  $\beta$ -actin were amplified in a ABI Prism 7700 Sequence detection system (PE Applied Biosystems), with denaturation at 95 °C for 10 min and 40 cycles of 15 s at 95 °C (melting) and 60 s at 58 °C (annealing and elongation). Unknown samples were quantified against a calibrator containing 10<sup>6</sup>–10 copies/reaction for hCG $\beta$ -3, -5, -8 and  $\beta$ -actin.

#### STATISTICAL ANALYSES

The clinical data were collected by an experienced epidemiologist (P.M.), all PCRs were performed by an experienced technician (J.J.T.M.H.), and statistical analyses were done by P.N.S.; all were blinded for other information. All measurements were performed blinded for clinical outcome. Statistical analyses were carried out with SPSS 10.0.5 software (SPSS Benelux BV). Differences in hCG $\beta$ -3, -5, -8 expression in samples divided in established categories were assessed with nonparametric Mann–Whitney *U*-tests or the Kruskal–Wallis test where appropriate. To analyze relationships between hCG $\beta$ -3, -5, -8 expression and various classic indices, we calculated two-sided asymptotic Pearson  $\chi^2$  values. Relapse-free survival (RFS) time (defined as the time from surgery until biopsy-confirmed diagnosis of recurrent disease) and overall survival (OS) time (defined as the time between date of surgery and death by any cause) were used as follow-up indices. Survival curves were generated using the method of Kaplan and Meier (16). Equality of survival distributions was tested using log-rank testing. The Cox proportional hazards model was used to assess the prognostic value of hCG $\beta$ -3, -5, -8 expression in addition to established factors that contributed to survival in univariate analysis (17). Two-sided *P* values <0.05 were considered statistically significant. Cases with >96 months of follow-up were censored at 96 months because of the rapidly decreasing number of patients thereafter.

### Results

#### $\beta$ -ACTIN AS HOUSEKEEPING GENE

In a preliminary study, we assessed the concurrent associations of 13 different housekeeping genes in 20 different

**Table 1. Categorical distributions of baseline characteristics of patients and hCG $\beta$ -3, -5, -8/ $\beta$ -actin ratios.**

| Variable                  | Total group of patients (n = 129) |      | Median (interquartile range) hCG $\beta$ -3, -5, -8/ $\beta$ -actin ratio, <sup>a</sup> $\times 10^4$ | <i>P</i> <sup>b</sup> |
|---------------------------|-----------------------------------|------|---|-----------------------|
|                           | n                                 | %    |   |                       |
| Age, years                |                                   |      |   |                       |
| <35                       | 3                                 | 2.3  | 4.5 <sup>c</sup>  |                       |
| $\geq 35$                 | 126                               | 97.7 | 4.1 (13)  | 0.605                 |
| Nodal status              |                                   |      |   |                       |
| Negative                  | 50                                | 38.8 | 4.4 (11)  |                       |
| Positive                  | 74                                | 57.4 | 3.6 (14)  | 0.945                 |
| Menopausal status         |                                   |      |   |                       |
| Premenopausal             | 39                                | 30.2 | 4.5 (13)  |                       |
| Postmenopausal            | 90                                | 69.8 | 3.8 (11)  | 0.593                 |
| Tumor type                |                                   |      |   |                       |
| Ductal                    | 98                                | 76.0 | 4.2 (13)  |                       |
| Lobular                   | 19                                | 14.7 | 5.4 (1.7)   |                       |
| Other/Unknown             | 12                                | 9.3  | 1.6 (4.0)   | 0.263                 |
| Tumor size, cm            |                                   |      |   |                       |
| $\leq 2$                  | 25                                | 19.4 | 4.1 (14)  |                       |
| >2                        | 103                               | 79.8 | 4.5 (12)  | 0.935                 |
| Histologic grade          |                                   |      |   |                       |
| I                         | 6                                 | 4.7  | 4.4 (17)  |                       |
| II/III                    | 55                                | 42.7 | 8.2 (7.1)   |                       |
| Unknown                   | 68                                | 52.7 | 4.2 (10)  | 0.325                 |
| ER, fmol/mg protein       |                                   |      |   |                       |
| <10                       | 49                                | 38.0 | 5.2 (18)  |                       |
| $\geq 10$                 | 78                                | 60.5 | 2.6 (8.4)   | 0.050                 |
| PgR, fmol/mg protein      |                                   |      |   |                       |
| <10                       | 57                                | 44.2 | 4.5 (12)  |                       |
| $\geq 10$                 | 71                                | 55.0 | 3.1 (13)  | 0.462                 |
| Surgery                   |                                   |      |   |                       |
| Mastectomy                | 103                               | 79.8 | 5.3 (10)  |                       |
| Breast-saving procedure   | 26                                | 20.2 | 3.8 (14)  | 0.885                 |
| Adjuvant radiotherapy     |                                   |      |   |                       |
| None                      | 43                                | 33.3 | 3.2 (5.4)   |                       |
| Any                       | 83                                | 64.3 | 4.6 (13)  | 0.183                 |
| Adjuvant systemic therapy |                                   |      |   |                       |
| None                      | 70                                | 54.3 | 4.5 (12)  |                       |
| Endocrine                 | 34                                | 26.4 | 3.1 (16)  |                       |
| Chemotherapy              | 10                                | 7.8  | 5.6 (27)  |                       |
| Both                      | 15                                | 11.6 | 2.2 (4.0)   | 0.247                 |

<sup>a</sup> Because of missing values, data do not always add up to 129.

<sup>b</sup> *P* denotes the statistical significance of differences between categories (Mann–Whitney *U*-test or Kruskal–Wallis test).

<sup>c</sup> Because of limited number of patients, no interquartile range is given for patients <35 years of age.

breast cancer samples.  $\beta$ -Actin was one of several genes that showed constant expression and was thus considered well suited for use as the housekeeping gene in the present study (results to be presented in detail elsewhere). We therefore normalized hCG $\beta$ -3, -5, -8 expression against  $\beta$ -actin expression.

**Table 2. Associations between hCG $\beta$ -3, -5, -8/ $\beta$ -actin ratios and clinicopathologic factors.**

| Variable                  | Median hCG $\beta$ -3, -5, -8/ $\beta$ -actin ratio <sup>a</sup> |                                      | P (Pearson $\chi^2$ ) |
|---------------------------|--|--------------------------------------|-----------------------|
|                           | <4.2 $\times$ 10 <sup>-4</sup>                                   | $\geq$ 4.2 $\times$ 10 <sup>-4</sup> |                       |
| Age, years                |  |                                      |                       |
| <35                       | 1 (33.3%)  | 2 (66.6%)                            | 0.578                 |
| $\geq$ 35                 | 63 (50.0%)   | 63 (50.0%)                           |                       |
| Nodal status              |  |                                      |                       |
| Negative                  | 24 (48.0%)   | 26 (52.0%)                           | 0.947                 |
| Positive                  | 36 (48.6%)   | 38 (51.4%)                           |                       |
| Menopausal status         |  |                                      |                       |
| Premenopausal             | 19 (48.7%)   | 20 (51.3%)                           | 0.786                 |
| Postmenopausal            | 45 (50.0%)   | 45 (50.0%)                           |                       |
| Tumor type                |  |                                      |                       |
| Ductal                    | 47 (48.0%)   | 51 (52.0%)                           | 0.237                 |
| Lobular                   | 8 (42.1%)  | 11 (57.9%)                           |                       |
| Other/Unknown             | 9 (75.0%)  | 3 (25.0%)                            |                       |
| Tumor size, cm            |  |                                      |                       |
| $\leq$ 2                  | 14 (56.0%)   | 11 (44.0%)                           | 0.810                 |
| >2                        | 49 (47.6%)   | 54 (52.4%)                           |                       |
| Histologic grade          |  |                                      |                       |
| I                         | 5 (83.3%)  | 1 (16.7%)                            | 0.229                 |
| II/III                    | 26 (47.3%)   | 29 (52.7%)                           |                       |
| Unknown                   | 33 (48.5%)   | 35 (51.5%)                           |                       |
| ER, fmol/mg protein       |  |                                      |                       |
| <10                       | 19 (38.8%)   | 30 (61.2%)                           | 0.119                 |
| $\geq$ 10                 | 43 (55.1%)   | 35 (44.9%)                           |                       |
| PgR, fmol/mg protein      |  |                                      |                       |
| <10                       | 25 (43.9%)   | 32 (56.1%)                           | 0.402                 |
| $\geq$ 10                 | 38 (53.5%)   | 33 (46.5%)                           |                       |
| Surgery                   |  |                                      |                       |
| Mastectomy                | 51 (49.5%)   | 52 (50.5%)                           | 0.893                 |
| Breast-saving procedure   | 13 (50.0%)   | 13 (50.0%)                           |                       |
| Adjuvant radiotherapy     |  |                                      |                       |
| None                      | 24 (55.8%)   | 19 (44.2%)                           | 0.286                 |
| Any                       | 38 (45.8%)   | 45 (54.2%)                           |                       |
| Adjuvant systemic therapy |  |                                      |                       |
| None                      | 31 (44.3%)   | 39 (55.7%)                           | 0.092                 |
| Endocrine                 | 18 (52.9%)   | 16 (47.1%)                           |                       |
| Chemotherapy              | 3 (30.0%)  | 7 (70.0%)                            |                       |
| Both                      | 12 (80.0%)   | 3 (20.0%)                            |                       |

<sup>a</sup> Values are given as n (%). Because of missing values, data do not always add up to 129.

#### hCG $\beta$ -3, -5, AND -8 mRNA CONCENTRATIONS IN BREAST CANCER TISSUES

The ratios of hCG $\beta$ -3, -5, -8 to  $\beta$ -actin in 129 breast cancer tissues ranged from 0 to  $5.3 \times 10^{-2}$  (median,  $4.2 \times 10^{-4}$ ). Nineteen (14.7%) samples showed no discernable amplification after 40 rounds of PCR. The hCG $\beta$ -3, -5, -8/ $\beta$ -actin ratios were examined for association with established clinicopathologic indices, as summarized in Table 1. The hCG $\beta$ -3, -5, -8/ $\beta$ -actin ratios were borderline significantly ( $P = 0.050$ ) associated with ER status, with ER-positive tumors exhibiting lower hCG $\beta$ -3, -5, -8/ $\beta$ -actin ratios. We found no other associations.

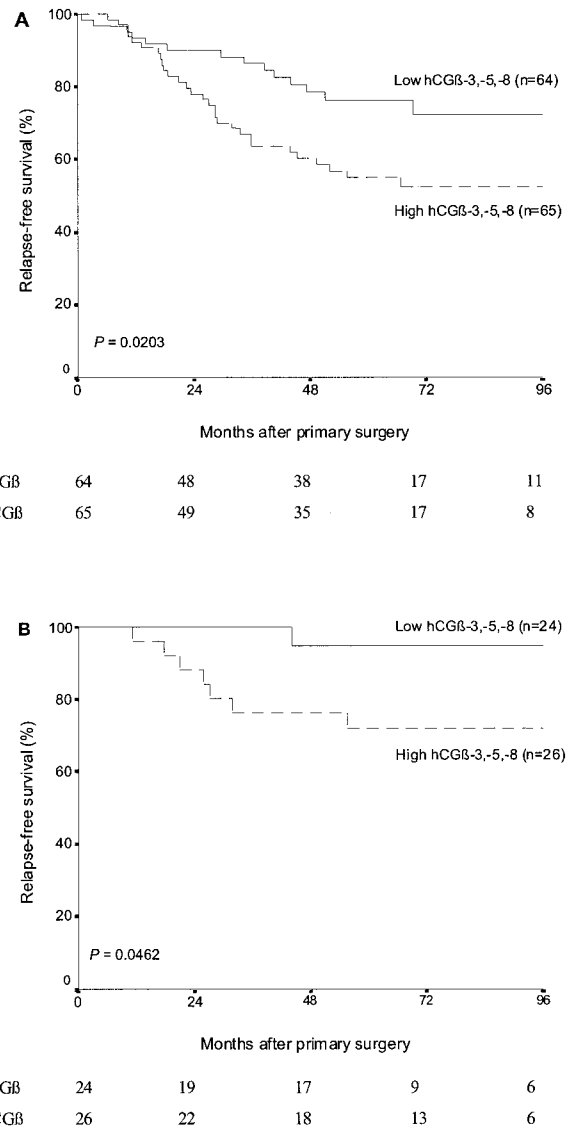


Fig. 1. RFS rates in breast cancer patients whose tumors expressed either low ( $<4.2 \times 10^{-4}$ ; solid line) or high ( $\geq 4.2 \times 10^{-4}$ ; dashed line) hCG $\beta$ -3, -5, -8/ $\beta$ -actin ratios.

(A), total group of 129 breast cancer patients; (B), node-negative subset of patients ( $n = 50$ ). The number of patients at risk at a certain time point is shown at the bottom of each panel.

#### hCG $\beta$ -3, -5, -8 EXPRESSION AND OUTCOME

In the group of 129 breast cancer patients, 43 patients had a recurrence during follow-up. At the time of analyses, 32 patients had died. To assess the association between hCG $\beta$ -3, -5, -8 expression and outcome, hCG $\beta$ -3, -5, -8/ $\beta$ -actin ratios were divided into equal sample sizes, i.e., at the median cutoff of  $4.2 \times 10^{-4}$ . We assessed the relationships between these dichotomized hCG $\beta$ -3, -5, -8 values and the clinicopathologic indices (Table 2) and found no significant associations with established indices.

At the median cutoff of  $4.2 \times 10^{-4}$ , hCG $\beta$ -3, -5, -8/ $\beta$ -actin ratios had prognostic significance for RFS (Fig. 1A). In univariate analysis, only nodal status ( $P = 0.0007$ ), size

**Table 3. Univariate (log-rank) and multivariate (Cox regression) analyses of RFS and OS in 129 breast cancer patients.**

| Variable   | P          |              | Hazard ratio<br>(95% CI) <sup>a</sup> |
|--|------------|--------------|---------------------------------------|
|  | Univariate | Multivariate |                                       |
| <b>RFS</b>   |            |              |                                       |
| Nodal status, positive vs negative   | 0.0007     | 0.001        | 3.62 (1.67–7.85)                      |
| Tumor size, >2 cm/≤2 cm  | 0.0391     | 0.114        |                                       |
| Age, <35 years/≥35 years   | 0.1729     |              |                                       |
| Menopausal status, pre-/post-  | 0.1011     |              |                                       |
| Tumor type, ductal/other   | 0.8392     |              |                                       |
| Histologic grade, III/II vs I  | 0.7492     |              |                                       |
| ER status, <10/≥10 fmol/mg protein   | 0.3318     |              |                                       |
| PgR status, <10/≥10 fmol/mg protein  | 0.5277     |              |                                       |
| Radiotherapy, yes/no   | 0.2230     |              |                                       |
| Adjuvant therapy, yes/no   | 0.8062     |              |                                       |
| hCG $\beta$ -3, -5, -8/ $\beta$ -actin ratio, ≥4.2 × 10 <sup>-4</sup> / $<$ 4.2 × 10 <sup>-4</sup> | 0.0203     | 0.015        | 2.25 (1.17–4.34)                      |
| <b>OS</b>  |            |              |                                       |
| Nodal status, positive vs negative   | 0.0078     | 0.012        | 3.17 (1.29–7.79)                      |
| Tumor size, >2 cm/≤2 cm  | 0.0771     | 0.204        |                                       |
| Age, <35 years/≥35 years   | 0.1621     |              |                                       |
| Menopausal status, pre-/post-  | 0.7643     |              |                                       |
| Tumor type, ductal/other   | 0.4883     |              |                                       |
| Histologic grade, III/II vs I  | 0.5210     |              |                                       |
| ER status, <10/≥10 fmol/mg protein   | 0.7324     |              |                                       |
| PgR status, <10/≥10 fmol/mg protein  | 0.5542     |              |                                       |
| Radiotherapy, yes/no   | 0.2667     |              |                                       |
| Adjuvant therapy, yes/no   | 0.5477     |              |                                       |
| hCG $\beta$ -3, -5, -8/ $\beta$ -actin ratio, ≥4.2 × 10 <sup>-4</sup> / $<$ 4.2 × 10 <sup>-4</sup> | 0.4525     |              |                                       |

<sup>a</sup> CI, confidence interval.

of the tumor ( $P = 0.0391$ ), and hCG $\beta$ -3, -5, -8/ $\beta$ -actin ratios ( $P = 0.0203$ ) were significantly associated with RFS in this group of breast cancer patients (Table 3). Patients whose tumors had low hCG $\beta$ -3, -5, -8 mRNA concentrations had a mean RFS time of 78.8 months, whereas patients with tumors with high concentrations had a mean RFS time of 64.1 months (Fig. 1A). At the median cutoff value, hCG $\beta$ -3, -5, -8 mRNA concentrations did not show a statistically significant prediction for OS ( $P = 0.4525$ ; Table 3).

In Cox regression multivariate analysis (Table 3) with nodal status and tumor size, hCG $\beta$ -3, -5, and -8 maintained their prognostic value for RFS, with values ≥ 4.2 × 10<sup>-4</sup> predicting shorter RFS ( $P = 0.015$ ; hazard ratio, 2.25; 95% confidence interval, 1.17–4.34). In multivariate analysis, only nodal status was statistically significant in predicting OS (Table 3).

The association of hCG $\beta$ -3, -5, -8 expression with prognosis of RFS was investigated in the subset of patients with node-negative disease in our group ( $n = 50$ ). At the median cutoff for the total group, we found a significant ( $P = 0.0462$ ) difference in prognosis despite the low number of events ( $n = 8$ ) in this group. Only 1 of 24 (4.1%) node-negative patients with low hCG $\beta$ -3, -5, -8 expression eventually relapsed, in contrast to 7 of 26 (26.9%) with high expression (Fig. 1B).

## Discussion

In this study, we show that the malignancy-associated hCG $\beta$ -3, -5, -8 transcripts have independent prognostic value for RFS in sporadic breast cancer patients. These results concur with and confirm an earlier study in 99 primary breast cancers in which a qualitative assay was used to assess the expression of these transcripts (9). In both the present study and that from Bièche et al. (9), no association with other established clinicopathologic indices was found. Thus, establishing hCG $\beta$ -3, -5, -8 expression values in the primary tumor can identify a group of breast cancer patients who are at higher risk for recurrence of the disease.

The role of hCG in cancer prognosis has been related to its protective effect of the fetoplacental unit against the immune system during pregnancy (18). The involvement of hCG in recurrence of disease reported here could also be related to its stimulatory effect on estradiol formation in breast cancer (19), by which hCG expression could lead to autocrine induction of increased growth of hormone-sensitive cancer cells or refractoriness to estrogen ablation treatment. Alternatively, gonadotropins have been found to induce vascular endothelial growth factor production in ovarian cancer (20) and thus indirectly stimulate angiogenesis. In our group of patients, however, we found no association of hCG $\beta$ -3, -5, -8 expression and vascular

endothelial growth factor mRNA or protein (data not shown).

Remarkably, of the four functional genes encoding the hCG  $\beta$ -subunit (11), the hCG $\beta$ -7 gene encodes an alanine (GCC) at position 117 as opposed to an aspartic acid (GAC) encoded by the hCG $\beta$ -3, -5, and -8 genes. The latter genes are reportedly involved in malignant transformation (8). Healthy breast tissue expresses only hCG $\beta$  subunits from the hCG $\beta$ -7 gene (8,9), and total hCG $\beta$  expression has no prognostic value (21). With the assay described here, we could confirm that healthy breast tissue did not express hCG $\beta$ -3, -5, or -8 transcripts in samples obtained from cytoreductive surgery (not shown) and confirm the reported prognostic value of the hCG $\beta$ -3, -5, and -8 genes. Considering the small difference in protein sequence, it is unclear why the hCG $\beta$ -3, -5, and -8 genes are specifically associated with malignancy and should have prognostic value for RFS in breast cancer. Possibly, the expression of this subset of hCG $\beta$  genes is regulated by factors that are also involved in the expression of genes associated with recurrence of breast cancer.

One candidate factor is Ets-2, a member of the Ets transcription factor family, which is characterized by a common DNA-binding domain (22). Ets-2 is infrequently up-regulated in prostate cancer (23), as is hCG $\beta$ -3, -5, -8 expression (6,8). The Ets-2 gene resides on chromosome 21 and is duplicated in Down syndrome (trisomy 21). In Down syndrome, placental hCG $\beta$ -5 expression is up-regulated (24), possibly because of duplication of the Ets-2 gene (25). Ets-2 is involved in protein kinase A-stimulated hCG $\beta$ -5 expression (25) and is also involved in urokinase-type plasminogen activator and matrix metalloproteinase-9 gene expression (26). Urokinase-type plasminogen activator and matrix metalloproteinase-9 are matrix-degrading enzymes with prognostic value in breast cancer (27,28).

Another possibility is promoter methylation patterns. Overall DNA methylation is decreased in breast carcinomas, playing a potentially important role in tumor development (29). The same holds true for the hCG $\beta$  locus on 19q13.3 in the placenta and choriocarcinoma (30). These tissues reportedly express only hCG $\beta$ -3, -5, and -8 (8), suggesting that hypomethylation of this site leads to preferential expression of the hCG $\beta$ -3, -5, and/or -8 genes. Thus, the prognostic value of hCG $\beta$ -3, -5, -8 expression could be an indication of the state of DNA hypomethylation rather than a direct effect of hCG $\beta$ -3, -5, and -8 protein on disease outcome. Indeed, several studies have indicated that in prostate cancer, in which expression of hCG $\beta$ -3, -5, or -8 does not occur on malignant transformation (6), DNA hypermethylation is a frequent mechanism leading to inactivation of key regulatory genes such as E-cadherin,  $\pi$ -class glutathione S-transferase, the tumor suppressors CDKN2 and PTEN, and insulin-like growth factor-II [for a review, see Ref. (31)]. However, overall, global DNA hypomethylation could also play an important role in prostate cancer progression (32). To the

best of our knowledge, the extent of DNA methylation at the hCG $\beta$  locus in breast or prostate cancer is not known.

To differentiate between the two types of gene transcripts, authors of previous studies used nested PCR, with a total of 50 amplification rounds, and fluorescently labeled primers that differed in their 3' nucleotides (9). Such an assay could be very sensitive but prone to false positives because of the high degree of amplification. Furthermore, the specificity of allele-specific primers differing in one nucleotide is difficult to assess, especially for primers with a 3' adenosine (33). The assay used in the current study has the benefits of real-time fluorescence reverse transcription-PCR, i.e., the assay is linear over a wide range of template concentrations. Furthermore, because of the closed-tube format, these assays are much less sensitive to false positives than previous reverse transcription-PCR assays. Finally, a probe, modified from the Molecular Beacon principle of Tyagi and Kramer (13), was devised that is highly specific for the hCG $\beta$ -3, -5, and -8 transcripts (6). The assay used in the current study thus is quantitative, sensitive, specific, and linear over a wide range of template concentrations. The fact that both assays yield similar results demonstrates the validity of this finding with respect to the prognostic value of hCG $\beta$ -3, -5, -8 expression in breast cancer.

It is of critical importance to identify the 30% of breast cancer patients with node-negative disease whose disease eventually will progress despite a relatively good prognosis based on the nodal status (34). If these patients could be identified, they could be treated with more aggressive therapy to prevent relapse, whereas the other 70% could be spared unnecessary treatment. Here we show that in the small subset ( $n = 50$ ) of node-negative patients in our group, hCG $\beta$ -3, -5, -8 expression has prognostic value for RFS. Only 1 of 24 patients with low hCG $\beta$ -3, -5, -8 expression relapsed as opposed to 7 of 26 patients with high expression. However, we assessed only a small group of patients, and the low number of events ( $n = 8$ ) that occurred during follow-up in these patients, associated with their good prognosis, impaired the significance of this finding. A larger group of patients, with possibly longer follow-up to assure a sufficient number of events, is necessary to confirm these preliminary results.

In summary, we show here that information on the expression of particular hCG $\beta$  genes in the primary tumor offers independent prognostic information on the RFS of breast cancer patients. Sensitive methods such as real-time, fluorescent reverse transcription-PCR are becoming more readily available. Robust standardization and technical validation is necessary for these techniques to be introduced for patient or treatment selection. The finding, however, of new genes with prognostic value, such as the hCG $\beta$ -3, -5, and -8 genes reported here, may shed more light on the epigenetic events associated with malignant transformation.

We gratefully acknowledge the surgeons, medical oncologists, and pathologists of the contributing hospitals for their contributions to this study: University Medical Center Nijmegen (Nijmegen, The Netherlands); Ziekenhuis Apeldoorn (Apeldoorn, The Netherlands); Deventer Ziekenhuis (Deventer, The Netherlands); Nieuw Spitaal (Zutphen, The Netherlands); and Streekziekenhuis Zevenaar (Zevenaar, The Netherlands). We also acknowledge Doorlène van Tienoven for assistance with collecting and archiving the breast tumor samples.

### References

- Bates SE, Longo DL. Tumor markers: value and limitations in the management of cancer patients. *Cancer Treat Rev* 1985;12:163–207.
- Mann K, Saller B, Hoermann R. Clinical use of hCG and hCG  $\beta$  determinations. *Scand J Clin Lab Invest Suppl* 1993;216:97–104.
- Marcillac I, Troalen F, Bidart JM, Ghillani P, Ribrag V, Escudier B, et al. Free human chorionic gonadotropin  $\beta$  subunit in gonadal and nongonadal neoplasms. *Cancer Res* 1992;52:3901–7.
- Iles RK, Persad R, Trivedi M, Sharma KB, Dickinson A, Smith P, et al. Urinary concentration of human chorionic gonadotrophin and its fragments as a prognostic marker in bladder cancer. *Br J Urol* 1996;77:61–9.
- Sheaff MT, Martin JE, Badenoch DF, Baithun SI.  $\beta$ hCG as a prognostic marker in adenocarcinoma of the prostate. *J Clin Pathol* 1996;49:329–32.
- Span PN, Thomas CM, Heuvel JJ, Bosch RR, Schalken JA, vd Loch L, et al. Analysis of expression of chorionic gonadotropin transcripts in prostate cancer by quantitative TaqMan and a modified Molecular Beacon RT-PCR. *J Endocrinol* 2002;172:489–95.
- Agnantis NJ, Patra F, Khaldi L, Filis S. Immunohistochemical expression of subunit  $\beta$  hCG in breast cancer. *Eur J Gynaecol Oncol* 1992;13:461–6.
- Bellet D, Lazar V, Bieche I, Paradis V, Giovangrandi Y, Paterlini P, et al. Malignant transformation of nontrophoblastic cells is associated with the expression of chorionic gonadotropin  $\beta$  genes normally transcribed in trophoblastic cells. *Cancer Res* 1997;57:516–23.
- Bieche I, Lazar V, Nogues C, Poynard T, Giovangrandi Y, Bellet D, et al. Prognostic value of chorionic gonadotropin  $\beta$  gene transcripts in human breast carcinoma. *Clin Cancer Res* 1998;4:671–6.
- Giovangrandi Y, Parfait B, Asheuer M, Olivi M, Lidereau R, Vidaud M, et al. Analysis of the human CGB/LHB gene cluster in breast tumors by real-time quantitative RT-PCR assays. *Cancer Lett* 2001;168:93–100.
- Policastro PF, Daniels-McQueen S, Carle G, Boime I. A map of the hCG  $\beta$ -LH  $\beta$  gene cluster. *J Biol Chem* 1986;261:5907–16.
- Lazar V, Diez SG, Laurent A, Giovangrandi Y, Radvanyi F, Chopin D, et al. Expression of human chorionic gonadotropin  $\beta$  subunit genes in superficial and invasive bladder carcinomas. *Cancer Res* 1995;55:3735–8.
- Tyagi S, Kramer FR. Molecular beacons: probes that fluoresce upon hybridization. *Nat Biotechnol* 1996;14:303–8.
- EORTC Breast Cancer Cooperative Group. Revision of standards for the assessment of hormone receptors in human breast cancer. *Eur J Cancer* 1980;16:1513–6.
- Tsourkas A, Behlke MA, Bao G. Structure-function relationships of shared-stem and conventional molecular beacons. *Nucleic Acids Res* 2002;30:4208–15.
- Kaplan EL, Meier P. Non-parametric estimation from incomplete observations. *J Am Stat Assoc* 1958;53:457–81.
- Cox DR. Regression models and life-tables (with discussion). *J R Stat Soc B* 1972;34:187–220.
- Acevedo HF, Tong JY, Hartsock RJ. Human chorionic gonadotropin- $\beta$  subunit gene expression in cultured human fetal and cancer cells of different types and origins. *Cancer* 1995;76:1467–75.
- Tanaka Y, Kuwabara K, Okazaki T, Fujita T, Oizumi I, Kaiho S, et al. Gonadotropins stimulate growth of MCF-7 human breast cancer cells by promoting intracellular conversion of adrenal androgens to estrogens. *Oncology* 2000;59:19–23.
- Wang J, Luo F, Lu JJ, Chen PK, Liu P, Zheng W. VEGF expression and enhanced production by gonadotropins in ovarian epithelial tumors. *Int J Cancer* 2002;97:163–7.
- Reimer T, Koczan D, Muller H, Friese K, Krause A, Thiesen HJ, et al. Human chorionic gonadotrophin- $\beta$  transcripts correlate with progesterone receptor values in breast carcinomas. *J Mol Endocrinol* 2000;24:33–41.
- Wasylyk B, Hahn SL, Giovane A. The Ets family of transcription factors. *Eur J Biochem* 1993;211:7–18.
- Liu AY, Corey E, Vessella RL, Lange PH, True LD, Huang GM, et al. Identification of differentially expressed prostate genes: increased expression of transcription factor ETS-2 in prostate cancer. *Prostate* 1997;30:145–53.
- Goshen R, Gonik B, Ariel I, Weiss Y, de-Groot N, Hochberg A. High levels of maternal serum human chorionic gonadotropin in Down syndrome pregnancies: the possible role of a transcription factor on chromosome 21. *Fetal Diagn Ther* 1999;14:106–11.
- Johnson W, Jameson JL. Role of Ets2 in cyclic AMP regulation of the human chorionic gonadotropin  $\beta$  promoter. *Mol Cell Endocrinol* 2000;165:17–24.
- Watabe T, Yoshida K, Shindoh M, Kaya M, Fujikawa K, Sato H, et al. The Ets-1 and Ets-2 transcription factors activate the promoters for invasion-associated urokinase and collagenase genes in response to epidermal growth factor. *Int J Cancer* 1998;77:128–37.
- Pacheco MM, Nishimoto IN, Mourao Neto M, Mantovani EB, Brentani MM. Prognostic significance of the combined expression of matrix metalloproteinase-9, urokinase type plasminogen activator and its receptor in breast cancer as measured by Northern blot analysis. *Int J Biol Markers* 2001;16:62–8.
- Look MP, van Putten WL, Duffy MJ, Harbeck N, Christensen IJ, Thomssen C, et al. Pooled analysis of prognostic impact of urokinase-type plasminogen activator and its inhibitor PAI-1 in 8377 breast cancer patients. *J Natl Cancer Inst* 2002;94:116–28.
- Soares J, Pinto AE, Cunha CV, Andre S, Barao I, Sousa JM, et al. Global DNA hypomethylation in breast carcinoma: correlation with prognostic factors and tumor progression. *Cancer* 1999;85:112–8.
- Campaign JA, Gutkin DW, Cox GS. Differential DNA methylation of the chorionic gonadotropin  $\beta$ -subunit multigene family. *Mol Endocrinol* 1993;7:1331–46.
- Rennie PS, Nelson CC. Epigenetic mechanisms for progression of prostate cancer. *Cancer Metastasis Rev* 1998–1999;17:401–9.
- Santourlidis S, Florl A, Ackermann R, Wirtz HC, Schulz WA. High frequency of alterations in DNA methylation in adenocarcinoma of the prostate. *Prostate* 1999;39:166–74.
- Ayyadevara S, Thaden JJ, Shmookler Reis RJ. Discrimination of primer 3'-nucleotide mismatch by Taq DNA polymerase during polymerase chain reaction. *Anal Biochem* 2000;284:11–8.
- Fisher B, Bauer M, Wickerham DL, Redmond CK, Fisher ER, Cruz AB, et al. Relation of number of positive axillary nodes to the prognosis of patients with primary breast cancer. An NSABP update. *Cancer* 1983;52:1551–7.